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Release of thrombomodulin from endothelial cells by concerted action of TNF-α and neutrophils: *in vivo* and *in vitro* studies

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SUMMARY

Inflammatory cytokines decrease the expression of thrombomodulin (TM) on the endothelial cell surface by suppression of TM transcription and translation or internalization with subsequent degradation. Nevertheless, elevated serum TM levels are found in diseases associated with systemical or locally increased levels of inflammatory cytokines. To study directly the *in vivo* effects of tumour necrosis factor-α (TNF-α) we determined the course of serum TM after systemic recombinant human (rh)TNF-α therapy. The TM levels were determined by enzyme-linked immunosorbent assay (ELISA). Systemic rhTNF-α therapy resulted in a marked and significant increase of serum TM. Using a mouse model we studied whether increased serum TM is associated with a decreased expression of TM on the endothelial surface *in vivo*. The immunohistochemical staining of the vasculature of meth-A sarcoma transplanted in mice showed a loss of TM immunoreactivity 4 hr after intravenous TNF-α application. To study the mechanism of TNF-α mediated release of TM, cultured endothelial cells were incubated with neutrophils and TNF-α. Incubation with TNF-α alone did not lead to an increase of TM *in vitro*. However TM was released into the culture supernatant when endothelial cells pretreated with TNF-α were exposed to neutrophils. This was associated with morphological evidence of endothelial cell damage. Therefore, the concerted action of cytokine-stimulated endothelial cells and neutrophils results in release of TM from cultured endothelial cells after rhTNF-α therapy. This might explain the increased serum TM levels observed in diseases associated with increased systemic or local levels of inflammatory cytokines despite the induced internalization and the direct inhibitory effects of TNF-α on TM transcription and translation.

INTRODUCTION

Thrombomodulin (TM) is an endothelial cell transmembrane glycoprotein and an important anti-coagulant as a receptor for thrombin. Bound to TM, thrombin changes its substrate specificity, leading to accelerated activation of protein C by the TM–thrombin complex.[1–4] TM is predominantly expressed on vascular endothelial cells of arteries, veins and capillaries, as well as on lymphatic endothelial cells and syncytiothrophoblasts.[5] Furthermore, TM is found on activated smooth muscle cells under certain conditions, mesothelial cells and malignant cells.[6–9] Small amounts of TM have been detected in platelets, megacaryocytes, monocytes and neutrophils.[3,9–11] A soluble form of TM is found in serum, plasma and urine, in addition to the membrane-bound glycoprotein,[12–15] which is most probably released due to endothelial cell damage.[16] The amount of TM expressed on the cell surface is influenced by tumour necrosis factor-α (TNF-α). TNF-α induces procoagulant activities of endothelial cells, while decreasing the expression of TM *in vitro*.[17–21]

Either the suppression of TM transcription and translation or the internalization with subsequent degradation of TM has been suggested as a mechanism for the reduced expression of TM on the endothelial cell surface.[21–25] Therefore, a decrease in serum TM should be expected in diseases with an activated immune system. However, in contrast to these *in vitro* observations, patients suffering from diseases with increased systemic or local levels of inflammatory cytokines and TNF-α (e.g. malaria or vasculitic disorders like systemic lupus erythematosus; SLE) show increased serum TM levels.[26–30] Therefore, to solve the question of increased TM levels despite decreased synthesis and expression, we studied the effect of

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Abbreviations: ; E-selectin, ELAM-1 (CD62E); HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1 (CD54); IL, interleukin; TM, thrombomodulin; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1 (CD106).

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TNF-α on the release of TM *in vivo* and *in vitro*. Furthermore, we studied possible mechanisms *in vitro* resulting in release of TM from cultured endothelial cells, possibly explaining elevated serum TM levels in diseases associated with an activated immune system.

MATERIALS AND METHODS

Patients

Successive serum samples from six patients receiving a total of 19 courses of intravenous (i.v.) recombinant human (rh)TNF-α therapy over 3–5 days were investigated. A serological follow up was available of one patient treated intra-arterially with rhTNF-α by an isolated limb perfusion.

All patients were treated according to protocols of phase II clinical trials, which were approved by the Ethics Committee of the University of Heidelberg, Germany. Before the start of therapy, informed consent was obtained from all patients. All patients had histologically defined advanced metastatic malignancies, either with progressive tumors under conventional chemotherapy, or with progressive malignant ascites under intensified standard therapies. The patients had received no chemotherapeutical treatment 3 weeks before therapy, had normal or maximal grade I impairment (WHO criteria) of haematological, hepatic and renal functions, and no evidence of other severe non-malignant diseases. Recombinant human TNF-α was supplied by KNOLL AG (Ludwigshafen, Germany). For the prevention of side-effects (e.g. chills, fever) under rhTNF-α therapy, the patients received paracetamol (1000 mg as suppositories) 30 min prior to the therapy and after a further 4 and 8 hr if required. In addition, all patients treated with systemic application of rhTNF-α received 30 mg pethidine before the start of therapy.

Altogether six patients received 19 courses of i.v. rhTNF-α therapy combined with subcutaneous interferon-α2 (IFN-α2; Roferon-A18®, Hoffmann La Roche, Grenzach-Wyhlen, Germany). According to the protocol of a phase II clinical trial, the patients were treated with 120 $\mu g/m^2$ body surface rhTNF-α (diluted in 250 ml 0·9% saline supplemented with 0·5% human serum albumin) as continuous i.v. infusion over 2 hr daily for 3–5 consecutive days. In addition, they received 5×10^6 U IFN-α2 on each second day for 3 weeks. If applicable, this therapy course was repeated after 21 days. The mean age of these patients was 55 years (range 51–65 years; four men, two women; one colon cancer, one peritoneal mesothelioma, one breast cancer, three renal cancers). Serum samples were collected before the start of a course of therapy and at 3, 6, 12 and 24 hr after start of the application of rhTNF-α therapy daily. The biological activity of this systemically applicated rhTNF-α was shown recently by increased levels of adhesion molecules (ICAM-1, E-selectin and VCAM-1), interleukin-6 (IL-6) and TNF-α receptors.[31] The clinical efficacy of the combined i.v. rhTNF-α therapy was poor, as observed previously by others.[32] Only one patient had stable disease over four courses of therapy.

According to the protocol of Lienard *et al.*[33] one female patient (70 years) with an advanced and, to conventional chemotherapy, refractory malignant melanoma of the left limb was treated by an isolated intra-arterial perfusion of the limb. A total of 150 μg rhTNF-α was given to the circulating perfusate over 90 min. Peripheral serum samples were obtained before the start of perfusion and after 0·5, 1, 2, 3, 8 and 24 hr. Histologically, a delayed and only temporary tumor cell necrosis was found.[31]

As control serum samples of 18 healthy white laboratory staff members (mean age 33 years, range 23–38 years, 11 male, seven female) were tested.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated according to the method of Jaffe *et al.*,[34] with modifications as described previously.[34–37] In brief, HUVEC were obtained from human umbilical cord veins by collagenase digestion (0·14%, collagenase from *Clostridium histolyticum*; Boehringer Mannheim, Mannheim, Germany), washed and cultured in tissue culture plates coated with gelatine (0·2% containing 1 μg/ml polymyxin B; Sigma Diagnostics, Deisenhofen, Germany). They were grown in medium 1640 [containing 200 mM L-glutamine, 20% fetal calf serum (FCS; Seromed® Biochrom KG, Berlin, Germany), 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma Diagnostics)]. For experiments the second passage of endothelial cells was used cultured in six-well plates, where the cells had been confluent for 2 days before the experiments started.

Before the start of experiments the medium was changed to a serum-free one (RPMI-1640, containing 200 mM L-glutamine and 50 U/ml penicillin/50 μg/ml streptomycin). Thereafter, as indicated in the Results, half of the wells were incubated with rhTNF-α (10 ng/ml medium) for 4 hr, washed twice with phosphate-buffered saline (PBS) and incubated with new medium. To some of the wells 2×10^6/ml neutrophils were added for 2 hr. To remove non-adherent neutrophils the medium was changed after 2 hr and the incubation was continued for 3 hr. The harvested culture supernatant was immediately stored at −20° for further investigations after each experimental step. In addition, the cells were harvested and lysed in Nonidet P-40 [NP-40; 1·5%, Calbiochem, La Jolla, CA; in 20 mM Tris–HCl, pH 7·4, 100 mM NaCl, 10 mM EDTA, 2 mM phenyl-methyl-sulphonyl fluoride (PMSF) and 0·6 mM leupeptin (Sigma Diagnostics)]. Determination of soluble (s)TM was performed in each of the supernatants collected. The experiments were performed in triplicate and repeated three times.

The required neutrophils were obtained from human peripheral blood of healthy white volunteers by venipuncture. Neutrophils were isolated from 100 ml of anti-coagulated EDTA blood by Polymorphprep® gradient centrifugation, according to the manufacturer's instruction (Immuno, Heidelberg, Germany; 1500 *g* for 30 min). Residual erythrocytes were removed by hypotonic lysis (0·2% NaCl for 20 seconds, then addition of 1·6% NaCl and centrifugation 1500 *g* for 10 min). Thereafter, cells were resuspended in RPMI-1640 supplemented with 10% FCS with a concentration of 2×10^7 neutrophils/ml.

For the assessment of neutrophil-mediated endothelial cell injury HUVEC were grown to confluency on gelatine-coated six-well plates in serum-free RPMI-1640 (containing 200 mM L-glutamine and 50 U/ml penicillin/50 μg/ml streptomycin). The monolayers were washed with PBS and incubated with rhTNF-α (10 ng/ml medium) or medium as required, for 4 hr. After washing with PBS again, some wells were incubated with 2×10^6/ml neutrophils. The wells were incubated for 10 hr, until

visually strong endothelial damage was detected microscopically. Then the monolayers were washed three times with cold PBS, air dried, fixed with 2% neutral buffered formaldehyde, and stained with haematoxylin and eosin using standard procedures.

TM ELISA

Soluble TM levels of serum, cell culture supernatants or lysed cells were determined by a two-site enzyme-linked immunoabsorbent assay (ELISA; Asserachrom thrombomodulin ELISA kit, Diagnostica Stago, Asnières, France), as described previously.[26] The ELISA was performed with the pre-coated plates provided according to the manufacturer's instructions, and measured at 492 nm with an automated ELISA plate reader (Titertek Multiscan Plus MKII, ICN/Flow Co., Meckenheim, Germany). The dilutions used were 1:5 for serum samples and 1:3 for cell culture supernatants. The concentrations were calculated in relation to the standard curves and the mean of duplicates taken.

Tissues

To examine immunohistologically the influence of TNF-α on the TM expression *in vivo*, C_3H mice were injected intradermally with meth-A sarcoma cells (10^6 cells/animal). After the tumors were grown to about 0·5 cm size, 5 μg rhTNF-α or 5 μg heat-inactivated rhTNF-α/mouse were injected intravenously via the tail vain. The mice were killed as indicated in the Results. The meth-A sarcoma tissues were frozen in a mixture of isopentane and dry ice immediately and stored in liquid N_2 using standard procedures. The experiments were repeated four times.

Immunohistochemistry

Four-micrometre thick cryostat sections of the meth-A sarcoma tissues were fixed in acetone at 4° for 10 min. They were washed in PBS and incubated with a rabbit anti-mouse TM IgG antibody (kindly provided by Professor D. Stern, New York, NY) with a dilution of 1:200 in a moist chamber for 60 min. After further incubation with a second peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma Diagnostics, dilution 1:100), the peroxidase activity was visualized by aminoethylcarbazol (Sigma ImmunoChemicals, Deisenhofen, Germany) using standard procedures.

Statistical analysis

If not stated otherwise, the mean and standard error are given. For the assessment of significance, a two-tailed Student's *t*-test was used for the *in vivo* data ($P < 0·05$) and the Wilcoxon paired difference test was used for the *in vitro* experiments ($P < 0·05$).

RESULTS

Because inflammatory cytokines like TNF-α and IL-1 reduce the expression of TM in endothelial cells, we expected a decrease of serum TM after an infusion of rhTNF-α. However, during 19 courses of systemic i.v. rhTNF-α therapy in six patients we found a marked and significant increase of soluble TM levels. During the course of therapy the consecutive rhTNF-α applications resulted in further augmented increases of serum TM, leading to an undulating pattern (Fig. 1a). In all

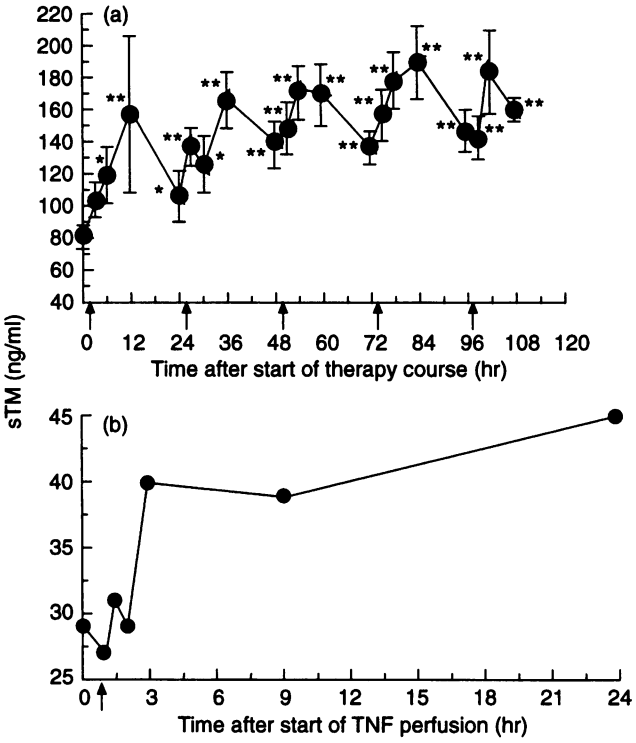

Figure 1. After rhTNF-α therapy a marked and significant increase of soluble TM levels was found due to i.v. [a; mean ± SEM, $n = 19$)] as well as due to intra-arterial application (b). The intravenous rhTNF-α was given 0, 24, 48, 72 and 96 hr after the start of a course of therapy for 2 hr (indicated by arrow), leading to an undulating increase of TM. The intra-arterial rhTNF-α was given over 90 min (indicated by arrow). $*P < 0·01$, $**P < 0·001$.

patients the serum TM values returned to the individual basal level before the beginning of the next course of therapy. Compared to the control values (serum TM 35·2 ± 3·2, range 18–52 ng/ml, $n = 18$), the basal level of serum TM in these patients was significantly increased (sTM 80·3 ± 4·7, range 47–115 ng/ml, $n = 19$, $P < 0·001$). Furthermore, an increase of serum TM was not only observed after i.v. but also after intra-arterial application. One patient was treated with an isolated intra-arterial limb perfusion with rhTNF-α. After a delay of 2–3 hr the TNF-α infusion led to an increase in serum TM levels (Fig. 1b).

Therefore, systemic administration of rhTNF-α resulted in increased serum TM levels, despite the known inhibitory effect of TNF-α on transcription and translation. To establish the mechanism of TM release by TNF-α we first studied its effect in an *in vivo* mouse model. After intradermal injection of meth-A sarcoma cells in mice, tumors of about 0·5 cm in size were studied immunohistochemically. Two hours as well as 4 hr after i.v. injection of 5 μg TNF-α/mouse, but not after injection with heat-inactivated TNF-α, a substantial loss of vascular cell-surface TM was observed compared with tumor sections of untreated mice (Fig. 2).

Taking these data together, systemic TNF-α application resulted in a simultaneous loss of TM from the endothelial cell surface and an increase in serum TM levels. Therefore, these results raise the question of whether TNF-α might act on

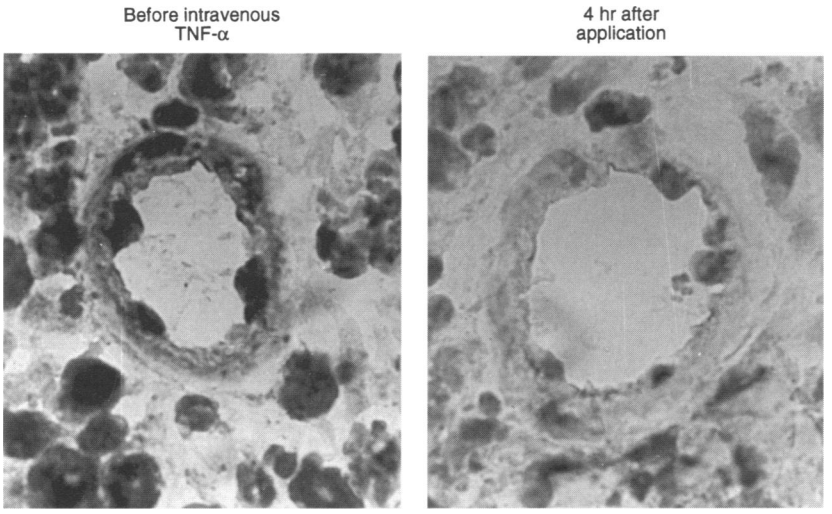

Figure 2. Immunohistochemical staining of thin sections of a meth-A sarcoma using a rabbit anti-mouse TM antibody documented the marked reduction of TM of the vascular endothelial cells 4 hr after i.v. application of a total of 5 μg rhTNF-α/mouse. Endothelial cells outside of these tumour sections stained more weakly for TM (not shown). The experiments were repeated four times (magnification ×40). In addition to the endothelial cells meth-A sarcoma cells were stained due to the known ability of tumor cells to express TM *de novo*.

cultured endothelial cells directly, leading to release of TM into the culture supernatant. However, when endothelial cells were incubated with TNF-α alone no morphological changes or significant increases in TM in the supernatant occurred. Similar data were obtained when endothelial cells were incubated with neutrophils alone (Table 1). This suggested that TNF-α might act in concert with other cells in order to release TM. As actions of neutrophils are involved in many inflammatory diseases associated with locally or systemically elevated TNF-α levels, we additionally studied the effects of neutrophils on activated endothelial cells *in vitro*. When endothelial cells, activated after a rhTNF-α pretreatment, were incubated with neutrophils the morphology of cells was greatly altered and TM released into the culture supernatant (Fig. 3 and Table 1). Furthermore, there was a significant difference in the cellular TM content, as determined in the lysed cells at the end of the *in vitro* experiment (TM in the 2×10^6 cells of medium and the medium neutrophil control: 229 ± 11 and 233 ± 11 ng versus 175 ± 9, $P < 0{\cdot}01$, and 162 ± 9 ng, $P < 0{\cdot}01$, in the TNF pretreated cells further incubated with medium or neutrophils, respectively, $n = 3$). The given TM values were corrected for the neutrophil-derived TM in all *in vitro* experiments involving neutrophils (mean TM 19 ± 5 ng, $n = 3$). Thus a concerted action of cytokine-activated endothelial cells and neutrophils resulted in TM release into the supernatant, potentially explaining the TNF-α-mediated increase in serum TM despite its inhibitory effect on TM transcription and translation.

DISCUSSION

TNF-α is an important mediator of inflammatory reactions associated with vascular changes, in addition to a broad spectrum of further biological activities.[38–44] Recently several investigators found an association of increased serum TM levels with various diseases with elevated systemic or local levels of inflammatory cytokines such as TNF-α (e.g. malaria or vasculitic disorders like SLE).[26–30,45] As TNF-α leads *in vitro* to increased internalization and lysosomal degradation,[21–24]

Table 1. Release of TM (ng) in culture supernatant (2×10^6 cells)

Pre-treatment period with	Medium control		Neutrophils and medium	
	Medium	TNF-α (1 nM)	Medium	TNF-α (1 nM)
After 4 hr	22·3 ± 2·6	19·6 ± 2·8	20·3 ± 3·1	18·3 ± 2·6
Incubation period with	Medium	Medium	Neutrophils	Neutrophils
After 2 hr	21·1 ± 3·2	17·1 ± 2·8	23·2 ± 2·6	34·3 ± 2·7*
After 5 hr	35·1 ± 4·3	32·1 ± 4·3	33·3 ± 4·5	74·4 ± 12·9*

The experiments were performed in triplicate and repeated three times.
* Significant difference to control values ($P < 0{\cdot}05$).

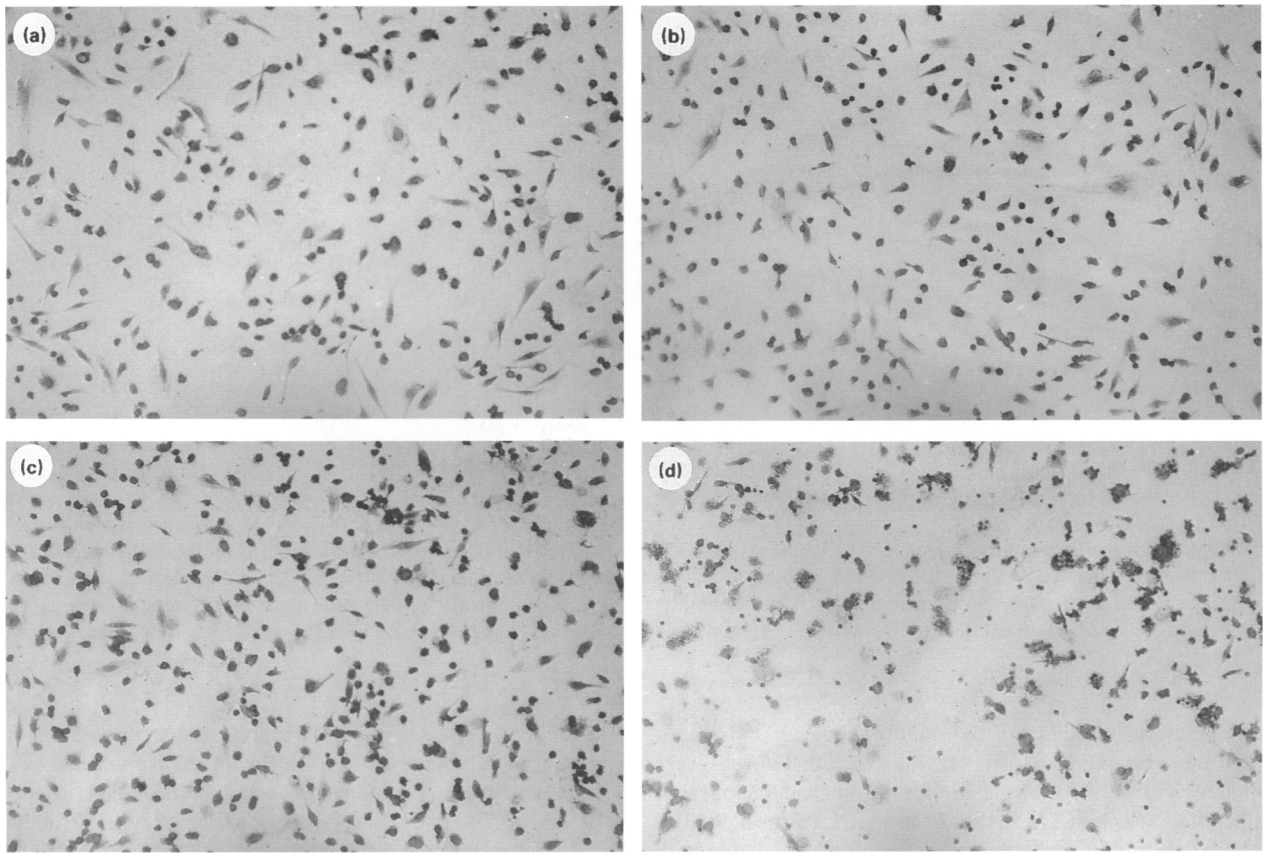

Figure 3. The histochemical staining (haematoxylin and eosin) of endothelial cells showed marked morphological cell changes only after a combination of rhTNF-α pretreatment with subsequent neutrophil incubation (d). The incubation of endothelial cells with medium (a), TNF-α (b) or neutrophils alone (c) did not result in a visible change of the endothelial cell monolayer (magnification ×40). The experiments were repeated five times.

inhibition of TM transcription and translation,[24,25] and increased procoagulant activity,[16–21] we asked how TNF-α might lead to an increase rather than decrease in serum TM. To study this we had the advantage of investigating the serum TM levels in patients taking part in a clinical study determining the anti-tumor effect of rhTNF-α therapy.[31] The systemic rhTNF-α application resulted in a marked and significant increase of serum TM levels *in vivo*. However, the current *in vitro* models provide no data to explain the TM increase in patient sera after rhTNF-α administration.

As the increase of serum TM was found more rapidly (2–3 hr after the start of application) than the inhibition of TM transcription and translation *in vitro*,[24,25] an alternative mechanism might be involved. In the *in vivo* animal model we were able to show that infusion of TNF-α resulted in a loss of TM from the endothelial cell surface of the vasculature supplying the tumor, before the TNF-α-mediated loss of cell-surface TM due to inhibition of translation became important. This raised the question of how TNF-α is involved in the release of TM from the endothelial cell surface.

Therefore, the effect of TNF-α was studied on cultured endothelial cells. However, no increase of TM in the supernatant was found when TNF-α was added to the culture alone. *In vitro* soluble TM is regarded as a marker of endothelial cell membrane injury, as it has been shown that the release of TM into the culture supernatant is directly time and dose related to hydrogen peroxide treatment of the cells, leading to cellular damage, and is independent from co-incubation with thrombin, the physiological ligand of TM.[16] Neutrophils are a main source of hydrogen peroxide, whereby *in vitro* neutrophil-mediated cell damage is found on cytokine-activated endothelial cells due to radical burst and after prolonged incubation due to activation of proteases.[46–54] TNF-α pretreatment of endothelial cells results in increased expression of adhesion molecules (e.g. ICAM-1, E-selectin, VCAM-1), leading to augmented cell adhesion and contact[55–59] with resulting increases of soluble adhesion molecules levels,[31,60] whereby the proliferating, angiogeneic and tumour-derived endothelial cells have a marked increased cytokine-dependent sensitivity.[41,61,62]

Because hydrogen peroxide leads to a TM increase in endothelial cell culture supernatant, we investigated the concerted action of neutrophils on TNF-α-activated endothelial cells *in vitro*. Our *in vitro* data indicate that neither TNF-α nor neutrophils alone but the interaction of neutrophils with TNF-α-activated endothelial cells leads to release of TM in the culture supernatant, concomitant with morphologically evident endothelial cell damage. However, our data do not prove that hydrogen peroxide is the only mediator of relevance released.

The fast return of serum TM to basal levels points to a short-acting effect of systemically administered rhTNF-α, which is consistent with the short detectable serum peak of

TNF-α and IL-6 after application and the unchanged susceptibility of the cells to rhTNF-α given during the repeated courses of therapy, leading to constantly repeated increases of soluble adhesion molecules *in vivo*.[31] The further observed elevated basal serum TM level might be due to increased alteration and damage of the vessels in rapidly growing malignant tissues, or due to an augmented liberation of TM from damaged tumor cells, of which some are known to express TM *de novo*.[7–9,63]

Altogether the *in vitro* data let us speculate that increased serum TM in patients with systemically or locally increased cytokine levels reflects a concerted action of neutrophils on cytokine-activated endothelial cells, resulting in cell damage. This mechanism could explain the surprising observation that TNF-α increases *in vivo* serum TM levels despite its inhibitory effect on TM translation and transcription and its induction of internalization. However, further studies in neutrophil-depleted animals are required to support this hypothesis.

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